IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Victor Raso

Application No.: 09/992,994

Filing Date: November 6, 2001

Title: IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*

Art Unit: 1652

Examiner: Patterson, C.

Docket No.: BBRI-2005

CERTIFICATE OF MAILING

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DECLARATION UNDER 37 CFR 1.131

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Victor Raso, declare and state as follows:

1. I am an inventor of the invention claimed in U.S. Application No. 09/992,994 filed November 6, 2001, with an effective filing date of June 16, 1999.

2. Prior to June 10, 1999, in laboratories at Boston Biomedical Research Institute (BBRI) in Boston, MA, I, the inventor named in the subject patent application, conceived of the invention of Claims 37-38, 40-42, 43 (as amended), 45, 48-51, 54, and 57-59 of the subject patent application. Documentary evidence that conception occurred before June 10, 1999 is provided in the attached Exhibit A. Exhibit A contains referenced pages from a true copy of a draft of the subject patent application, which was prepared prior to June 10, 1999. The referenced pages, along with the appended correspondence letter, are evidence that conception occurred prior to June 10, 1999. Applicant was diligent from just prior to June 10, 1999 to June 16, 1999, during which final revisions on the draft application were made and transmittal papers were prepared.

Evidence of conception of Claim 37 and 41 can be found on page 6, first full paragraph of Exhibit A. Evidence of conception of Claims 38 and 40 can be found on page 24, last paragraph of Exhibit A. Evidence of conception of Claim 42 can be found on page 6, last paragraph of Exhibit A. Evidence of conception of Claim 43, as amended, can be found on page 18, last paragraph of Exhibit A. Evidence of conception of Claim 45 can be found on page 6, first full paragraph of Exhibit A. Evidence of conception of Claim 48 can be found on page 23, first full paragraph of Exhibit A. Evidence of conception of Claims 49-51 can be found on page 18, first paragraph of Exhibit A. Evidence of conception of Claim 54 can be found on page 6, first full paragraph of Exhibit A. Evidence of Claim 57 can be found on page 6, first full paragraph and page 18, second paragraph of Exhibit A. Evidence of Claim 58 can be found on page 6, first full paragraph and page 18, third paragraph of Exhibit A. Evidence of conception of Claim 59 can be found on page 6, first full paragraph and page 18, last paragraph, also of Exhibit A.

3. The dates and other confidential information have been redacted in the above-referenced Exhibits.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful

false statements may jeopardize the validity of the application or any patent issued thereon.

Signature	
Name	Victor Raso, Ph.D.
Date	

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EXHIBIT A

FARRELL & ASSOCIATES, P.C.
12 Riverwood Drive
P.O. Box 999
York Harbor, Maine 03911

Boston Office
50 Congress Street
P.O. Box 2169
Boston, Massachusetts 02106
(617) 722-4044
Facsimile (617) 722-9344

(207) 363-0558
Facsimile (207) 363-0528

Kevin M. Farrell

Shayne Y. Huff, Ph.D.
Technical Specialist*

Richard L. Sampson
Of Counsel

Ms. Pamela Torpey
P.O. Box 981
Douglas, MA 01516

Re: BBRI-2004
Immunological Control of Beta-Amyloid Levels In Vivo

Dear Pamela:

Enclosed please find a draft patent application, prepared by Dr. Shayne Huff, based on the above-referenced invention disclosure. The application is in near-final form, but we have requested characterization of antibodies produced against molecules which mimic the transition states of the beta-amyloid peptide. We are claiming such antibodies, we understand that such antibodies have been produced, and we feel that from an enablement standpoint, this support would be very helpful. If gathering this information is unduly burdensome at this time we can, of course, file without it.

I have made a note to contact you next week regarding this case. We do not want to hold up the filing if the data referred to above will not be available in the very near term. Any comments from Dr. Raso can be incorporated in short order.

Best regards,

Kevin M. Farrell

KMF:tlw

cc: Victor Raso, Ph.D., w/enc.
Shayne Y. Huff, Ph.D., w/o enc.

BBRI\ARCH\2004.L1

DRAFT

IMMUNOLOGICAL CONTROL OF BETA-AMYLOID LEVELS IN VIVO

Background of the Invention

Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. These extracellular deposits are found in the neo-cortex, hippocampus and amygdala as well as in the walls of the meningeal and cerebral blood vessels. The principal component of these plaques is a 39 to 43 residue β-amyloid peptide. Each plaque contains approximately 20 fmole (80 picograms) of this 4 kDa peptide (Selkoe et al., *J. of Neurochemistry 46*: 1820 (1986)). Apolipoprotein E and neurofibrillary tangles formed by the microtubule-associated tau protein are also often associated with Alzheimer's disease.

β-amyloid is proteolytically cleaved from an integral membrane protein called the β-amyloid precursor protein. The gene which codes for this protein in humans is found on chromosome 21 (St George-Hyslop et al., *Science 235*: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). Numerous cultured cells and tissues (eg. brain, heart, spleen, kidney and muscle) express this β-amyloid precursor protein and also secrete the 4 kDa β-amyloid fragment into culture media, apparently as part of a normal processing pathway.

While it is difficult to establish an absolute causal relationship between β-amyloid or the plaques it forms and Alzheimer's disease, there is ample evidence to support the pathogenic role of β-amyloid. For example, patients with Down's syndrome have an extra copy of the β-amyloid precursor protein gene due to trisomy of chromosome 21 (St George-Hyslop et al., *Science 235*: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, early-onset familial Alzheimer's disease can result from mutations in the β-amyloid precursor protein gene which fall within or adjacent to the β-amyloid sequence (Hardy, J., *Nature Genetics 1*: 233 (1992)). These observations are consistent with the notion that

deposition of β-amyloid as plaques in the brain are accelerated by an elevation in its extracellular concentration (Scheuner et al., *Nature Med. 2:* 864 (1996)). The finding that β-amyloid is directly neurotoxic both *in vitro* and *in vivo* (Kowall et al. *Proc. Natl. Acad. Sci. 88*: 7247 (1991)), ~~seems~~ suggest ~~the possibility~~ that soluble aggregated β-amyloid, not the plaques per se, may produce the pathology.

Observations have indicated that amyloid plaque formation may proceed by a crystallization type mechanism (Jarrett et al., *Cell 73*: 1055 (1993)). According to this model, the seed that initiates plaque nucleation is an β-amyloid which is 42 or 43 amino acids long ($A\beta_{1-43}$). The rate-determining nucleus formed by $A\beta_{1-43}$ or $A\beta_{1-42}$ allows peptides $A\beta_{1-40}$ or shorter to contribute to the rapid growth of an amyloid deposit. This nucleation phenomenon was demonstrated *in vitro* by the ability of $A\beta_{1-42}$ to cause the instantaneous aggregation of a kinetically stable, supersaturated solution of $A\beta_{1-40}$. That finding has led to the possibility that $A\beta_{1-40}$ might be relatively harmless in the absence of the nucleation peptides $A\beta_{1-42}$ or $A\beta_{1-43}$. Indeed, elevated levels of these long peptides have been found in the blood of patients with familial Alzheimer's disease (Scheuner et al., *Nature Med. 2:* 864 (1996)). Moreover, $A\beta_{1-42}$ or $A\beta_{1-43}$ was found to be the predominant form deposited in the brain plaques of many Alzheimer's disease patients (Gravina et al., *J. of Biol. Chem. 270*: 7013 (1995)).

Given the central role played by β-amyloid, it has become increasingly important to understand the interrelationship between the different pools of these molecules in the body. Free β-amyloid present in the blood most likely arises from peptide released by proteolytic cleavage of β-amyloid precursor protein present on cells in the peripheral tissues. Likewise most of the free β-amyloid found in the brain and cerebrospinal fluid is probably derived from peptide released by secretase cleavage of β-amyloid precursor protein expressed on brain cells. The

peptides are identical regardless of origin, and the results from several studies suggest an intercommunication between these pools.

Brief Description of the Figures

Figure 1 is an amino acid sequence listing (SEQ ID NO: 1) of the 43 residue β-amyloid peptide (Aβ).

Figure 2 is an amino acid sequence listing (SEQ ID NO: 2) of the antigenic peptide made from the N-terminal sequence of ~~Aβ~~ β-amyloid ($A\beta_{1-16}$).

Figure 3 is an amino acid sequence listing (SEQ ID NO: 3) of the antigenic peptide made from the central region of ~~Aβ~~ β-amyloid ($A\beta_{10-25}$).

Figure 4 is an amino acid sequence listing (SEQ ID NO: 4) ($A\beta_{35-43}$) of the antigenic peptide made from the C-terminal sequence of ~~Aβ~~ β-amyloid

Figure 5 is a diagrammatic representation of data from an ELISA comparing monoclonal antibody binding to $A\beta_{35-43}$ and $A\beta_{1-43}$ versus $A\beta_{1-40}$.

Figure 6 indicates the amide linkages in the peptide made from the ~~Aβ~~ β-amyloid C-terminal sequence (SEQ ID NO: 4) that were independently replaced with a statyl moiety, to generate the different statine transition state analogs of the peptide.

Figure 7 indicates the amide linkages in the peptide made from the ~~Aβ~~ β-amyloid central sequence (SEQ ID NO: 3) that were independently replaced with a statyl moiety, to generate the different phenylalanine statine transition state analogs of the peptide.

Figure 8 is a structural comparison between the native ~~Aβ~~ β-amyloid peptide and the transition state phenylalanine statine ~~Aβ~~ β-amyloid peptide analog.

Figure 9 is a structural comparison between the native ~~Aβ~~ β-amyloid peptide and the reduced peptide bond transition state ~~Aβ~~ β-amyloid peptide analog.

Figure 10 is a formulaic representation of the native C-terminal region of β-amyloid, and the phosphonamidate transition state analog of

the C-terminal region of $A\beta$ ($A\beta_{35-43}$).

Figure 11 indicates the putative transition state for peptide hydrolysis by zinc peptidases, and the phosphonate and phosphonamidate mimics.

Figure 12 is a structural comparison of the native $A\beta$ peptide and the transition state phosphonamidate $A\beta$ peptide which has the peptide link between Gly 38 and Val 39 replaced with a phosphonamidate bond.

Figure 13 is a diagrammatic representation of data from an ELISA which assess the binding of monoclonal antibodies, generated to transition state $A\beta$ peptide analogs, to the normal $A\beta_{1-43}$ and to the phenylalanine statine transition state $A\beta$ peptide.

Figure 14 is a diagrammatic representation of data from an ELISA comparing antibody binding to the statine transition state $A\beta$ peptide versus native $A\beta_{1-43}$.

Figure 15 is a graph of data showing the cleavage of ^{125}I-$A\beta$-sepharose by monoclonal antibodies generated to transition state analogs of $A\beta$.

Figure 16 is a diagrammatic representation of data which indicates the attachment of bispecific antibody to receptor-positive cells.

Figure 17 is a diagrammatic representation of data indicating the transcytosis of vectorized bispecific antibody into brain.

Detailed Description of the Invention

The present invention relates to immunologically based methods for controlling levels of β-amyloid in the body of an animal. The invention is based on the finding that antibodies specific for β-amyloid are able to bind β-amyloid in the presence of a physiological level of human serum albumin. The invention is also based on the finding that an animal can tolerate the presence of antibodies specific for β-amyloid in amounts sufficient to sequester β-amyloid in the bloodstream.

One aspect of the present invention related to a method for sequestering free β-amyloid in the bloodstream of an animal. The soluble and insoluble forms of β-amyloid present within an animal are in dynamic equilibrium. Soluble β-amyloid is thought to translocate between blood and cerebrospinal fluid. Insoluble β-amyloid aggregates deposit from the soluble pool in the brain, as amyloid plaques. Results detailed in the Exemplification section below indicate that intravenous administration of antibodies specific for β-amyloid to an animal impedes the passage of soluble β-amyloid out of the peripheral circulation. This occurs because the β-amyloid specific antibodies, which are restricted to the peripheral circulation, bind to β-amyloid and sequester it in the circulation. Such sequestration is accomplished through intravenous administration of an appropriate amount of antibodies specific for β-amyloid to the animal. The amount of antibody ~~administered~~ which is sufficient to produce sequestration when administered is dependent upon various factors (e.g. specific characteristics of the antibody to be delivered, the size, metabolism, and overall health of the animal) and ~~should~~ one needs to be determined on a case by case basis.

Administered antibodies can be monoclonal antibodies, a mixture of different monoclonal antibodies, polyclonal antibodies, or any combination therein. In one embodiment, the antibodies bind to the C-terminal region of β-amyloid. Such antibodies specifically bind the less abundant, but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$. In another embodiment, a combination of antibodies having specificity for various regions of β-amyloid are administered. In another embodiment, antibodies which catalyze the hydrolysis of β-amyloid, discussed in more detail below, are administered either alone or in combination with other anti-β-amyloid antibodies. to which is administered the antibodies are administered

The animal is any animal which has circulating soluble β-amyloid. In one embodiement, the animal is a human. The human

may be a healthy individual, or alternatively, may be suffering from or at risk for a disease in which elevated β-amyloid levels are thought to play a role, for example a neurodegenerative disease such as Alzheimer's disease.

A related aspect of the present invention is a method for sequestering free β-amyloid in the bloodstream of an animal by ~~generating~~ an immune resonse within the animal to endogenous β-amyloid. The results detailed in the Exemplification below indicate that an animal can tolerate the induction of an immune response which produces antibodies to endogenous β-amyloid, and that the presence of such antibodies will alter the distribution of β-amyloid in the body, in a similar manner as the above described method of administering β-amyloid binding antibodies. The immune response to endogenous β-amyloid is generated by immunizing the animal with one or more antigens comprised of epitopes present on β-amyloid ~~endogenous to the animal~~. Epitopes present on the innoculated antigens can correspond to epitopes present on any region of the β-amyloid molecule. In a preferred embodiment, epitopes found on the C-terminal region of β-amyloid are used to generate antibodies which specifically bind the $A\beta_{1-43}$ species as opposed to the smaller $A\beta_{1-40}$. In an alternate embodiment, a combination of epitopes ~~and antigen containing epitopes~~ are administered to generate a variety of antibodies to β-amyloid. A more generalized immune response is generated by immunizing either with a mixture of different small peptide antigens or with the full-length 43 residue β-amyloid peptide. In another embodiment, antigens used for inoculation include transition state analogs of β-amyloid peptides to induce antibodies which have catalytic activity directed towards β-amyloid hydrolysis, described in detail below.

The immunoreactivity of the antigens can be enhanced by a variety of methods, many of which involve coupling the antigen to an immunogenic carrier. In addition, various methods are known and available to one of skill in the art for specifically

enhancing the immunogenicity of endogenous molecules or the epitopes contained therein. Various modifications can be made to the β-amyloid antigen(s) described herein to render it more compatible for human use. For example, the peptide(s), can be genetically engineered into appropriate antigenic carriers, or DNA vaccines can be designed.

The above techniques for sequestering β-amyloid in the circulation are also useful for reducing ~~brain β-amyloid levels~~ the levels of β-amyloid in the brain. Because the formation of amyloid plaques in the brain is dependent, at least in part, on the levels of free β-amyloid present in the brain, reducing brain β-amyloid levels of an animal will in turn reduce the formation of amyloid plaques in the brain. Therefore, the above techniques are ~~also~~ useful for preventing the formation of amyloid plaques in the brain of an animal. This is especially applicable to an animal which is considered at risk for the development of amyloid plaques; a risk which may result from a genetic predisposition or from environmental factors. Administration of antibodies, or immunization of the animal to produce endogenous antibodies, ~~for~~ to β-amyloid can be of therapeutic benefit to such an animal (e.g. a human who has a family history of Alzheimer's disease, or who is diagnosed with the disease).

Another aspect of the present invention relates to antibodies which are characterized by the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage. Experiments detailed in the Exemplification section demonstrate the generation of different antibodies which have proteolytic activity towards β-amyloid. Such antibodies are generated by immunizing an animal with an ~~β-amyloid peptide~~ antigen which is a transition state (or ~~analog~~) of the β-amyloid peptide. A transition state analog mimics the transition state that β-amyloid adopts during hydrolysis of a specific ~~predetermined~~ amide linkage. Transition state analogs useful for generating the catalytic antibodies include, without limitation, statine, phenylalanine statine, phosphonate, phosphonamidate, and

reduced peptide bond transition state analogs.

Antibodies generated to epitopes unique to the transition state preferentially bind β-amyloid in the transition state. Binding of these antibodies stabilizes the transition state, which leads to hydrolysis of the corresponding amide bond. The particular amide linkage to be hydrolyzed is chosen based upon the desired cleavage product. For example, cleavage of full length β-amyloid into two peptide fragments which cannot aggregate into amyloid plaques would be of therapeutic use in the methods disclosed herein. Monoclonal antibodies which recognize the transition state of specific amide likages in β-amyloid include ..... **[VR: PLEASE PROVIDE LIST OF AMIDE LINKAGES THAT THE MONOCLONAL ANTIBODIES RECOGNIZE.]**

At least two different classes of antibodies are generated by the above methods. The first class preferentially binds the transition state analog, and also detectably cross reacts with natural β-amyloid, when using an ELISA as detailed in the Exemplification section, to detect binding. The second class binds the transition state analog, and does not detectably cross react to natural β-amyloid, at levels which are detectable via using the ELISA procedure detailed in the Exemplification section to detect binding. (see the for specific ELISA procedure.) Both classes of antibodies have potential value as catalytic antibodies. These respective binding affinities of an anti-transition state characteristics of native vs. transition state are likely to reflect its activity at catalyzing) hydrolysis. It is thought that in order for an antibody to have activity at catlyzing hydrolysis of a protein, it must posess at least a minimal ability to bind the natural (non-transition) sp 7. state of the protein. Antibodies which retain significant binding for β-amyloid, (that strongly cross react with natural β-amyloid) may be more efficient at catalyzing hydrolysis due to a higher efficiency of binding the β-amyloid. Once bound, these antibodies force the protein into a transition state conformation for hydrolytic cleavage. Alternatively, antibodies which only minimally cross react with natural β-amyloid, although less

e.g. antibodies

catalysis activity of the antibody.

efficient at binding native β-amyloid, are likely to be more efficient at forcing the bound β-amyloid into the transition state conformation for hydrolytic cleavage. It should be pointed out that failure to detect binding of the anti-transition state antibodies to natural β-amyloid by the ELISA methods presented in the Exemplification herein does not necessarily reflect an inability to bind natural β-amyloid sufficiently to function as a catalytic antibody. More likely, a lack of detection merely reflects the sensitivity limitations of the assay.

Antibodies which bind substantial affinity for the predicted cleavage products of the native β-amyloid peptide may be subject to product inhibition and might therefore exhibit low turnover. Such undesireable antibodies can be identified by secondary screening using peptides which contain epitopes of the predicted cleavage products (e.g. via ELISA).

In a preferred embodiment, the antibodies are monoclonal. These can be produced by immunizing an animal (e.g. mouse, guinea pig, or rat) with the transition state analog antigen, and subsequently producing hybridomas from the animal, by standard procedures. Hybridomas which produce the desired monoclonal antibodies are then identified by screening. One example of a screening method is presented in the Exemplification section which follows. In another embodiment, the antibodies are polyclonal. Polyclonal antibodies are generated by immunizing an animal (e.g. a rabbit, chicken, or goat) with antigen and obtaining sera from the animal. Polyclonal antibodies which have the desired binding specificities can be further purified from the sera by one of skill in the art through the course of routine experimentation.

Another aspect of the present invention is the use of statine and reduced peptide bond analogs to elicit catalytic antibodies having proteolytic activity. The Exemplification section below details methods for using statine analogs as antigen in the production of catalytic antibodies, and also lists examples of anti-transition-state antibodies generated using these methods. The "statyl" moiety is derived from naturally

evolved protease transition state inhibitors like amastatin, pepstatin, and bestatin. These naturally-occurring statine-based inhibitors have been used to effectively block the activity of aminopeptidases, aspartic proteases and the HIV protease. Synthetic peptides containing a statine residue offer novel features for the induction of catalytic antibodies. The statyl moiety has a tetrahedral bond geometry, its length is extended by two CH_2 units, it has a strategically placed OH group and the structure has no charge. The presence of the additional CH_2 units is expected to elicit a more elongated antibody combining site, and antibodies generated to this site will induce extra strain on the peptide substrate, producing an accellerated catalysis. In addition, the OH group in these statine analogs is thought to better approximate the position and chemistry of the true transition state. Statine-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from the more commonly used negatively charged phosphonate analogs.

Reduced peptide bond analogs introduce a tetrahedryl configuration, without increasing the distance between amino acid residues. This feature should more closely approximate the true transition state geometry, than previously used analogs. A positively charged secondary amine replaces the amide nitrogen of the natural polypeptide and should elicit a complementary negatively charged side chain at a proximal locus in the antibody combining site. The presence of such ancillary glutamyl or aspartyl groups ~~present~~ on the antibody will ~~could~~ assist antibody-mediated catalysis of peptide cleavage via acid-base exchange. Reduced peptide bond-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from using the more commonly used negatively charged phosphonate analogs. Both reduced peptide bond analogs and ~~also~~ statine analogs can be used to produce transition state analog antigens ~~which mimic~~ to a wide variety of proteins or for a wide variety of specific polypeptides. These antigens can in turn be used to generate the respective catalytic antibodies.

Administration of the β-amyloid catalytic antibodies described above finds use in the above described methods for 1) sequestering free β-amyloid in the bloodstream of an animal, 2) reducing levels of β-amyloid in the brain of an animal, and 3) preventing the formation of amyloid plaques in the brain of an animal. Experiments presented in the Exemplification demonstrate that immunization of an animal with a transition state analog results in the generation of an immune response to produce antibodies which recognize the transition state, and which catalyze hydrolysis of the β-amyloid protein. This indicates that the transition state analogs can be used as antigens in these methods to induce the production of antibodies in the animal which recognize and catalyze cleavage of endogenous β-amyloid.

Methods which involve reducing overall levels of β-amyloid in an animal through the proteolytic action of the above described catalytic antibodies are also encompassed by the present invention. The presence of functional catalytic antibodies in the circulation of an animal reduces the level of β-amyloid in the circulation. Accordingly, the present invention provides a method for reducing levels of circulating β-amyloid in an animal by introducing the above described catalytic antibodies into the animal.

The present invention also provides a method for reducing levels of circulating β-amyloid in an animal by immunizing the animal with a β-amyloid transition state analog to induce antibody production. The use and design of such vaccines is described above, and detailed in the Exemplification section below. Administration of the antibodies to the animal is preferably via intravenous administration. Such antibodies are either monoclonal, mixed monoclonal, polyclonal or a mixture thereof. The origin of the antibody may affect the half-life of the antibody in the animal; antibodies from less related species are more likely to be recognized as foreign by the animal's immune system. Preferably, administered antibodies are derived from a species closely related to the animal, to maximize half-

life and minimize adverse reactions by the host. Administration of isolated variable region antibody fragments may produce beneficial results in this regard.

The reduction of β-amyloid levels in the circulation of an animal is expected to displace the equilibrium of β-amyloid in the body, and lead to a reduction in the levels of β-amyloid in the brain of the animal through mass action. In this respect, the present invention provides methods for reducing the levels of β-amyloid in the brain of an animal, by either administering catalytic antibodies to the animal, or by administering a transition state analog to induce endogenous antibody production. It follows that these procedures also have value as methods for preventing the formation of amyloid plaques in the brain of an animal, since the resulting reduction in the levels of β-amyloid in the brain of an animal should prevent the formation of amyloid plaques. These procedures also have value as methods for disaggregating amyloid plaques present in the brain of an animal, since evidence indicates that lower brain β-amyloid levels can lead to the disaggregation of plaques.

another aspect of The present invention ~~also~~ provides ~~for~~ a more direct method of altering the distribution of β-amyloid in the brain by actually delivering anti-β-amyloid antibodies to the brain. Methods described above for reducing levels of β-amyloid in the brain and for preventing aggregation of amyloid plaques depend upon exchange between β-amyloid pools in the circulation the cerebrospinal fluid, the exchange being driven by a disruption of the equilibrium between the pools. In contrast, delivery of anti-β-amyloid antibodies to the brain will directly affect β-amyloid aggregation. Evidence presented in the Exemplification section below indicates that the binding of certain anti-β-amyloid antibodies inhibits the initial aggregation of β-amyloid *in vitro*, and also disaggregates preformed *in vitro* β-amyloid complexes. Moreover, if insoluble peptide is in equilibrium with a low level of soluble β-amyloid, then an anti-β-amyloid binding

antibody could upset this balance and gradually dissolve the precipitate. These observations indicate that the presence of β-amyloid antibodies in the brain will directly inhibit the formation of amyloid plaques and will also disaggregate preformed plaques by disrupting the dynamic equilibrium between soluble β-amyloid and fibrillar β-amyloid deposited as plaques. Furthermore, a highly active catalytic antibody is expected to destroy insoluble β-amyloid plaques by hydrolytically cleaving the constituent aggregated peptides.

One way of delivering antibodies to the brain is by producing vectorized antibodies competent for transcytosis across the blood-brain barrier. Vectorized antibodies are produced by covalently linking an antibody to an agent which promotes delivery from the circulation to a predetermined destination in the body. Examples of such vectorized antibodies can be found in the prior art .... **[VR: PLEASE PROVIDE REFERENCES]** One such agent is another antibody which is directed towards a cell surface component, such as a receptor, which is transported away from the cell surface. Examples of antibodies which confer the ability to trancytose the blood-brain barrier include, without limitation, anti-insulin receptor antibodies, and also anti-transferrin receptors (Saito et al., *Proc Natl Acad Sci USA 92*: 10227-31 (1995); Pardridge et al., *The Primate 12*: 807-816 (1995); **[VR: PLEASE VERIFY THE ACCURACY OF THIS LAST REFERENCE AS IT WAS NOT CLEAR IN THE MATERIALS PROVIDED]** Broadwell et al., Exp Neurol 142: 47-65 (1996)). This first antibody is covalently linked to an antibody which binds β-amyloid. Alternatively, coupling the β-amyloid antibodies to ligands which bind these receptors (e.g. insulin, transferrin, or LDL **[VR: PLEASE PROVIDE COMPLETE NAME OF LDL]**) will also produce a vectorized antibody competent for delivery to the brain from the circulation (Descamps et al., *Am. J. Physiol. 270*: H1149-H1158 (1996); Duffy et al., *Brain Res. 420*: 32-38 (1987); Dehouck et al., *J. Cell Biol. 138*: 877-889 (1997)).

A vector moiety can be chemically (or genetically) attached to

the anti-β-amyloid antibody to facilitate its delivery into the central nervous system. This vector component can be for example, an anti-transferrin receptor antibody or anti-insulin receptor antibody which binds to those receptors on the brain capillary endothelial cells (Bickel et al., *Proc Natl Acad Sci U S A* 90: 2618-22 (1993); Pardridge et al., *J Pharmacol Exp Ther 259*: 66-70 (1991); Saito et al. *Proc Natl Acad Sci U S A* 92: 10227-31(1995); Friden et al., *J. Pharm. Exper. Ther.* 278: 1491-1498 (1996)) which make up the blood-brain barrier. The resulting bifunctional antibody (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., *J. Biol. Chem.* 272: 27618-27622 (1997); Raso, V. *Anal. Biochem.* 222:297-304 (1994); Raso et al., *Cancer Res* 41: 2073-2078 (1981); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)) will attach to the appropriate receptors on the luminal side of the vessel. Once bound to the receptor, both components of the bispecific antibody can pass across the blood-brain barrier by the process of transcytosis. Anti-β-amyloid antibodies which have entered the brain interact directly with both β-amyloid plaques and the soluble β-amyloid pool. It has been estimated that concentrations of macromolecules in the 10^{-8}-10^{-7} M range can be achieved in the brain using vector-mediated delivery via these brain capillary enriched protein target sites (Maness et al., *Life Sciences* 55: 1643-1650 (1994); Lerner et al., *Science* 252: 659-667 (1991)). Importantly, the vector appears safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody showed no loss of integrity of the blood-brain barrier, using a radioactive sucrose probe (Broadwell et al., *Exp Neurol 142:* 47-65 (1996)).

The Exemplification details the production of vectorized bispecific antibodies which bind β-amyloid. The bispecific

antibodies transcytose across the blood brain barrier via a first specificity which binds the transferrin receptor. Use of antibodies which bind the transferrin receptor for delivery of agents across the blood brain barrier is described by Friden et al. in U.S. Patents No. 5,182,107; No. 5,154,924; No. 5,833,988; and No. 5,527,527; the contents of which are incorporated herein by reference.

Results from experiments presented in the Exemplification section which follows indicate that the produced bispecific antibodies retain their separate specificities and are delivered across the blood-brain barrier into the brain parenchyma and brain capillaries of a live animal when administered intravenously.

Alternative methods for the production of bispecific antibodies have been described for genetically engineering bispecific reagents or for producing them intracellularly by fusing the two different hybridoma clones **[VR: PLEASE PROVIDE REFERENCES FOR THESE METHODS]**. Vectorized bispecific antibodies produced by these techniques can also be used in the methods of the present invention.

Since the introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells, it may be beneficial to produce and utilize smaller vectorized $F(ab')_2$ bispecific reagents. It has been shown that aggregated β-amyloid itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect could be greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need be added. Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents may provide for intracerebral

delivery. In addition, the two types of vectorized molecules may have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

Depending on their design, anti-β-amyloid bispecific antibodies ~~situated~~ in the brain ~~can~~ function ~~in~~ ~~three different~~ ways to reduce soluble β-amyloid and β-amyloid deposits. An anti-β-amyloid bispecific antibody that tightly binds soluble β-amyloid will not only sequester the peptide but, due to efflux of vectorized molecules from the central nervous system (Kang et al., *J. Pharm. Exp. Ther.* 269: 344-350 (1994)), ~~potentially can~~ carry the bound ~~As~~ out of the brain and release it into the blood stream. ~~This~~ clearance mechanism would lead to a continuous cycling of β-amyloid out of the brain.

To be effective the anti-β-amyloid sites of a bispecific antibody must be empty before passage out of the blood and into the brain. Therefore the concentration of bispecific antibody in animals must exceed the level of β-amyloid circulating in the blood. Calculations performed based upon known β-amyliod levels (Scheuner et al., *Nature Med. 2*: 864-870 (1996)) and a medium-range plasma level of bispecific antibody expected in a treated animal indicated 99.9% of the bispecific antibodies that enter the brain will have unoccupied anti-Aβ combining sites.

Another way of delivering antibodies to the brain is via direct infusion of anti-β-amyloid antibodies into the brain of an animal. This technique gives these antibodies immediate access to β-amyloid in the brain without having to cross the blood-brain barrier. Direct infusion can be accomplished via direct parenchymal or intracerebroventricular infusion (Knopf et al., *J Immunol. 161*: 692-701 (1998)). Briefly, the animal is anesthetized and placed in a stereotaxic frame. A midsaggital incision is made on the scalp to expose the skull and the underlying fascia is scraped away. A hole is drilled to accept a sterilized length of stainless steel hypodermic tubing, which is

stereotaxically advanced so that its tip is appropriately located in the brain. A guide cannula is then attached to the skull and sealed. The cannula remains in place for multiple infusions of antibody into the brain. A bolus of a sterile 50 mg/ml solution of a monoclonal anti-β-amyloid can be infused over a 2-8 minute period into an immobilized animal via an injection cannula.

Delivery of catalytic antibodies into the brain of an animal via one of the above described methods, can also be used to disaggregate amyloid plaques present in the brain. The advantage of delivering an β-amyloid-specific catalytic antibody into the brain is two-fold. The β-amyloid peptide is permanently destroyed by such antibodies and, since catalysis is continuous, each antibody inactivates many target β-amyloid molecules in the brain. Thus much less vectorized bispecific antibody has to be delivered into the central nervous system to achieve the desired depletion of β-amyloid.

The amount of antibody to be administered or delivered to the animal should be sufficient to cause a significant reduction in β-amyloid levels in the brain of the animal. The appropriate amount will depend upon various parameters (e.g. the particular antibody used, the size and metabolism of the animal, and the levels of endogenous β-amyloid) and ~~should be~~ is to be determined on a case by case basis. Such determination is within the means of one of ~~skill~~ average skill in the art through ~~the course of~~ no more than routine experimentation.

It is expected that additional benefits with respect to lowering brain β-amyloid levels and preventing or disaggregating amyloid plaques can be achieved through utilizing a combination of one or more of the above described approaches.

Exemplification

PART 1: RETENTION OF β-AMYLOID IN THE CIRCULATION

Synthesis of β-Amyloid Peptide Antigens

The amino acid sequence of the 43 residue β-amyloid peptide (Aβ) is listed in Figure 1. To determine which sites on this Aβ peptide were best suited for antibody-mediated therapy, three key regions (amino-terminal, central and carboxy-terminal) of the Aβ 43-mer were chosen to generate epitope-specific vaccines. These shortened peptides served as antigenic epitopes to induce a highly specific antibody response.

Monoclonal antibodies to the amino-terminal region of Aβ have been shown to have the ability to solubilize Aβ aggregates (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997))(Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93(1): 452 (1996)). A peptide consisting of the amino-terminal region of Aβ was similarly designed (shown in Fig. 2 and listed in SEQ ID NO: 2) and used to elicit amino-terminal specific antibodies that bind Aβ. A Cys residue was added to the C-terminus of the Aβ sequence to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein such as maleimide-activated Keyhole Limpet Hemocyanin (KLH).

A peptide encompassing the central region of Aβ was synthesized (shown in Figure 3 and listed in SEQ ID NO: 3). A Cys residue was placed at the N-terminus of the Aβ sequence to provide a sulfhydryl linkage group for coupling the peptide to antigenic maleimide-activated carrier proteins such as KLH.

To produce an antigen for eliciting an immune response directed against the carboxy-terminus of Aβ (Suzuki et al., *Science* 264:1336(1994)), a decapeptide encompassing the N-terminal region of Aβ, with an additional Cys residue at the N-terminus, was synthesized (Shown in Fig. 4, and listed in SEQ ID

NO: 4). The Cys substitution was designed to provide a sulfhydryl linkage group for coupling the peptide to antigenic maleimide-activated carrier proteins such as KLH.

Coupling the peptides to an antigenic carrier protein

The different Cys containing Aβ peptides were individually thioether-linked to maleimide-activated KLH. A multivalent Aβ vaccine was also produced by simultaneously linking all three of these peptides to maleimide-activated KLH. In addition the full-length Aβ 43-mer was linked to KLH using glutaraldehyde.

Antibodies Elicited with the β-Amyloid Vaccines

Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. The mice were either bled or sacrificed for removal of the spleen for hybridoma production. Sera and monoclonal antibodies obtained were characterized for binding to Aβ.

Table 1 shows the results from an ELISA run with 1/100 diluted serum from two non-immunized control mice versus 1/100 and 1/1000 diluted serum from a mouse that was immunized with a central region Aβ peptide-KLH vaccine. The free Aβ peptide was adsorbed directly onto the microtitre plate to avoid detection of anti-KLH antibodies in the serum. Monoclonal antibodies raised against this central region Aβ peptide have also been successfully identified using this assay.

Table 1 ELISA for Binding to the Central Region Aβ Peptide

Addition		Antibody Bound (O.D. 450nm)
Control Serum A	1/100	0.666
Control Serum B	1/100	0.527
Mouse 1 antiserum	1/100	3.465

Mouse 1 antiserum	1/1000	2.764

A binding assay was performed to determine whether the monoclonal anti-Aβ antibodies identified ~~by the above assays~~ also bound to the full length Aβ peptides. ^{125}I-Aβ_{1-43} probe was incubated with hybridoma secretions from the indicated clones. A standard polyethylene glycol separation method was used to detect ^{125}I-Aβ_{1-43} bound antibody (Table 2). Results presented in Table 2 indicate that the antibodies generated to the peptide fragments also bound full length Aβ_{1-43}.

Table 2 ^{125}I-Aβ_{1-43} Binding Assay

Addition		^{125}I-Aβ_{1-43} Bound (cpm)
Control Hy		3,171
Control Hy		2,903
6E2		15,938
6E2	1/10	9,379
3B1		12,078
3B1	1/10	3,353
8E3		10,789
8E3	1/10	3,249

This raises the concern that the ~~reported result~~ suggests that the ~~observed~~ bound albumin will interfere w/ anti-body binding.

It was recently reported that when ^{125}I-Aβ_{1-40} is added to human plasma, ~89% binds to albumin (Biere et al., *Journal of Biological Chemistry* 271(51):32916 (1996)). Binding assays were performed in the presence and absence of serum albumin, to determine whether albumin binding ~~would~~ interfere with antibody

binding to Aβ. The ability of purified 5A11 monoclonal anti-Aβ antibody to bind ^{125}I-Aβ_{1-40} was unaffected by the presence of human serum albumin (HSA) at 60 mg/ml, even though this was a 500-fold molar excess over the antibody concentration (Table 3). These results indicate that the ability of antibodies to bind to and sequester Aβ in the blood will not be attenuated by the presence of other binding proteins.

Table 3. ^{125}I-Aβ_{1-40} Binding to Antibody in the Presence of Human Serum Albumin*

Addition	^{125}I-Aβ_{1-40} Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79
Control + HSA*	3,102	-
+ 5A11 anti-Aβ + HSA*	55,304	75

*HSA at 60 mg/ml (~1 mM); anti-Aβ 5A11 at 2x10^{-6} M; Added ~70,000 cpm of ^{125}I-Aβ_{1-40}

Monoclonal Antibody Production

A mouse was immunized with a KLH conjugate of the central region phenylalanine statine transition state mimic of the central region Aβ_{10-25} peptide. A hybridoma fusion was performed and the resulting monoclonal antibodies analyzed to characterize the specificity of the immune response to the vaccine. Hybridoma supernatants produced in the fusion were screened using ELISA to assess their binding to the Aβ_{1-43} peptide.

The monoclonal antibodies produced were determined to bind to the Aβ_{1-43} peptide adsorbed directly onto an ELISA plate. Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while the addition of media alone produced low background color. These results indicate that the

antibodies not only bound to the small peptide immunogen but they were also reactive with the full-length $A\beta_{1-43}$. Importantly, antibodies bound to the carrier-free $A\beta$ peptide adsorbed directly onto microtitre plates, showing their specificity for the peptide rather than the immunogenic carrier. The high affinity 5A11 monoclonal antibody (Table 3) was obtained from this hybridoma fusion. **[VR: THIS IS MISLEADING. PLEASE RECTIFY THIS STATEMENT WITH THE FACT THAT ANTIBODY 5A11 WAS OBTAINED FROM IMMUNIZATION WITH A TRANSITION STATE MIMIC PEPTIDE ANTIGEN.]**

A second mouse was immunized with a KLH conjugate of the $A\beta_{35-43}$ analog encompassing the C-terminal region of $A\beta$. Serum from the mouse was screened for reaction with $A\beta_{1-43}$ adsorbed directly onto the ELISA wells. The assay results are presented in Table 4. The spleen of this mouse was then used for a hybridoma fusion to further characterize the specificity of its immune response. Importantly, none of the mice immunized with $A\beta$ vaccines or the anti-$A\beta$ ascites-producing mice displayed ill effects even though some of those induced antibodies cross-react with mouse $A\beta$ and mouse amyloid precursor protein.

Table 4 ELISA for Binding of Antiserum Directed to the Carboxy-terminal $A\beta$ Peptide

	Antibody Bound (O.D. 450nm)
Addition	**Native $A\beta_{1-43}$**
Control Serum	0.484
Mouse Antiserum	1.765

Monoclonal antibodies from hybridoma clones generated above were screened for binding to the small carboxy-terminal peptide $A\beta_{35-43}$ and the full-length $A\beta_{1-43}$. Results are presented in Figure 5. The monoclonal antibodies bound to the carboxy-

terminal locus on each of these carrier-free Aβ peptides adsorbed directly to the microtitre plate, confirming their specificity for the peptide rather than the immunogenic carrier. The clones were also tested with $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid residue version of Aβ and thus will specifically bind to the carboxy-terminus of $A\beta_{1-43}$ (Fig.5). Used therapeutically, this vaccine should elicit antibodies which will preferentially bind the less abundant but more noxious $A\beta_{1-43}$ species in the blood, as opposed to the smaller and less detrimental $A\beta_{1-40}$.

Antibodies Affect the Distribution of ^{125}I-Aβ in Normal Mice

Anti-Aβ antibodies in the circulation cannot cross the blood-brain barrier to a significant extent and therefore should act as a sink that prevents $^{125}I\text{-}A\beta_{1-40}$ from reaching the brain. This retention effect was demonstrated by measuring the blood levels in mice 4 h after injecting them with equal amounts of $^{125}I\text{-}A\beta_{1-40}$ either alone or along with our 5A11 anti-Aβ monoclonal antibody (Table 5). The passage of $^{125}I\text{-}A\beta_{1-40}$ out of the peripheral circulation was greatly curtailed in animals which concomitantly received the specific anti-Aβ antibody. That finding extends the *in vitro* results with the 5A11 antibody (Table 3) by demonstrating that it can also effectively bind Aβ in an experimental animal. The observation that treatment with this antibody resulted in 10-times more $^{125}I\text{-}A\beta_{1-40}$ in the circulation indicates that the equilibrium distribution of Aβ in the body can be dramatically altered by selective sequestration in the blood.

Table 5 Anti-Aβ Antibody Impedes the Passage of $^{125}I\text{-}A\beta_{1-40}$ Out of the Circulation

	$^{125}I\text{-}A\beta_{1-40}$ in Blood

Mouse Injected With	(cpm/gm)
^{125}I-Aβ_{1-40} alone	27,300
^{125}I-Aβ_{1-40} + 5A11 anti-Aβ	278,900

Materials and Methods

Peptide synthesis. The 40mer Aβ_{1-40}, the 43mer Aβ_{1-43}, and the three small Aβ peptides Aβ_{1-16}, Aβ_{10-25}, and Aβ_{35-43}, were synthesized by standard automated Fmoc chemistry. Newly synthesized peptides were purified by HPLC and their composition was verified by mass spectral and amino acid analysis. The Aβ 43mer was obtained from a commercial source (Bachem, Torrance, CA).

Conjugation of β-amyloid peptides to immunogenic carriers. The small Aβ peptides were linked to the KLH carrier protein in order to render them antigenic. A Cys residue was strategically placed at the N- or C-terminal end of these Aβ peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This linkage is stable and attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH is typically obtained by this conjugation method. The longer, full length Aβ peptides were linked to carrier proteins using a glutaraldehyde coupling procedure.

The outlined methods are an effective and expedient way of producing experimental vaccines for use in animals.

Immunization of Mice. Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. Briefly, mice were injected i.p. with antigen emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. The mice were i.v. boosted with antigen in PBS three days prior to bleeding them or removing the spleen for hybridoma fusions to produce monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of these ~~induced~~ antibodies cross-react with mouse Aβ and mouse amyloid precursor protein.

ELISA. The presence of bound anti-peptide antibodies was revealed by using a peroxidase-labeled anti-mouse IgG probe followed by the chromogenic substrate (Engvall et al., Immunochemistry 8: 871-875 (1971).

Binding Assay. Both $A\beta_{1-43}$ and $A\beta_{1-40}$ were radiolabeled with ^{125}I. The iodinated peptide was separated from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89:5462 (1992)). ^{125}I-$A\beta_{1-43}$ probe was incubated for 1h at 23°C with Hy media taken from hybridoma clones producing monoclonal anti-Aβ antibodies. A standard polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody.

~~PART~~ II: ELICITING MONOCLONAL ANTIBODIES WITH TRANSITION STATE ANTIGENS

Transition state peptide antigens

Different types of transition state peptide antigens were synthesized to use in the generation of antibodies which preferentially recognize (hydrolysis) transition states of Aβ at a predetermined amide linkage position.

A series of statine (Sta) transition state analogs encompassing the carboxy-terminal region of Aβ (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) were synthesized. Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40}, Val_{40} and Ile_{41} and Ile_{41} and Ala_{42}, with a "statyl" moiety (-CHOH-CH_2-CO-NH-) was designed to elicit

catalytic antibodies that hydrolytically cleave Aβ at one of these sites (Figure 6). A Cys residue was placed at the N-terminal position of these peptides to provide a suitable linkage group for coupling to a maleimide-activated carrier protein.

A series of phenylalanine statine (PhSta) transition state analogs encompassing the central region of Aβ (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/PhSta-Phe/PhSta-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory (Fig. 7).

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20} and between Phe_{20} and Ala_{21} with a statyl moiety (-CHOH-CH_2-CO-NH-) was designed to elicit catalytic antibodies that hydrolytically cleave Aβ at these sites (Figure 7). A Cys residue was placed at the C-terminus of these peptides to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins ~~such as KLH~~.

A structural comparison (Fig. 8) was made between the native Aβ peptide and the transition state phenylalanine statine Aβ peptide using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

The peptide link -CO-NH- between Phe_{19} and Phe_{20} was replaced with an elongated "statyl" moiety -CHOH-CH_2-CO-NH- and an energy minimization was applied. This orientation shows the difference between the planar peptide link -CO-NH- of natural Aβ (left) versus the extended, tetrahedral "statyl" moiety -CHOH-CH_2-CO-NH- in the transition state peptide (right).

An antibody combining site complementary to a tetrahedral statine transition state analog will force the planar peptide bond of the Aβ substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aβ at that locus in the peptide sequence.

A reduced peptide bond linkage can be easily placed at almost any site in the Aβ molecule to produce a reduced peptide bond transition state analog. This analog can also be used to

The possibility of using a reduced peptide bond linkage to mimic the ~~hydrolysis~~ transition state of hydrolysis of ~~the~~ an amide peptide bond [illegible] was also

elicit catalytic antibodies that will hydrolytically cleave Aβ at the chosen site. The first reduced peptide bond transition state Aβ analog made was the (Gln-Lys-Leu-Val-Phe-CH_2-NH_2^+-Phe-Ala-Glu-Asp-Val-Gly-Cys-amide) central region peptide; [calculated 1,342 (M+1); observed 1,344].

A structural comparison (Fig. 9) was made between the native Aβ peptide and the reduced peptide bond transition state Aβ analog using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation. The peptide link -CO-NH- between Phe_{19} and Phe_{20} was replaced with a reduced peptide bond -CH_2-NH_2^+- and an energy minimization was applied. The orientation shown indicates the difference between the planar peptide link -CO-NH- of natural Aβ (left) versus the corresponding tetrahedral moiety -CH_2-NH_2^+- in the reduced peptide bond transition state analog (right).

A phosphonamidate transition state analog of the carboxy-terminal region of Aβ has also been synthesized (Fig. 10). Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} with a phosphonamidate moiety (-PO_2^--NH-) was designed to elicit catalytic antibodies that will hydrolytically cleave Aβ at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures in Fig. 11 represent the putative transition state for peptide hydrolysis by zinc peptidases, and the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state intermediates are known to be formed by reaction with each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

The synthesis of phosphonate Aβ transition state analog peptide (eg. N-acetyl-Cys-Met-Val-Gly-Gly-PO_2^--O-Val-Val-Ile-Ala-amide) will follow a similar scheme and will use some of the same intermediates described for the phosphonamidate transition state

analog.

A structural comparison was made between the native Aβ peptide and the transition state phosphonamidate Aβ peptide (Fig. 12) using a graphics workstation. The peptide link -CO-NH- between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond -PO_2^--NH- and an energy minimization was applied. The orientation shown in Fig. 12 illustrates the difference between the planar peptide link -CO-NH- of native Aβ (left) versus the corresponding tetrahedral phosphonamidate bond -PO_2^--NH- in the transition state peptide (right).

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 12, will force the normally planar bond of the Aβ substrate peptide on the left into a transition state-like conformation. Such bond distortion was expected to catalyze the hydrolytic cleavage of the Aβ peptide at the Gly_{38}-Val_{39} linkage.

Immunization with transition state peptide antigens

Peptide antigens were coupled to the immunogenic carrier KLH prior to immunization of mice.

Standard protocols were used to immunize the Tg2576 transgenic mice and BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen (Fig. 7) and also the statine (Sta) transition state analogs encompassing the carboxy-terminal region of Aβ (Fig. 6) (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) **[SH:THESE WERE NOT THE ONLY TRANSITION STATE ANTIGENS USED TO GENERATE HYBRIDOMAS/MONOCLONAL ANTIBODIES. VR IS MAKING A TABLE CATALOGING**

THE DIFFERENT TS ANALOGS, SITES MODIFIED AND MABS GENERATED]

Demonstration of Aβ binding by generated antibodies

It was very important to demonstrate that the anti-Aβ and anti-transition state Aβ monoclonal antibodies bound to the natural $A\beta_{1-43}$ peptide which they were designed to sequester or cleave. To do this, $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide was separated from unlabeled material by HPLC. Probe was incubated with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. The amount of ^{125}I-$A\beta_{1-43}$ bound to antibody was determined using a polyethylene glycol separation method. Results of the experiment are presented in Table 6.

The data in Table 6 demonstrate the ability of the purified 5A11 monoclonal anti-Aβ antibody to bind a high percent of ^{125}I-$A\beta_{1-40}$. This binding assay was used to screen clones and purified antibodies (Table 6) for their ability to bind Aβ. Similar procedures can also serve as the basis for a competitive displacement assay to measure the relative binding strength of different unlabeled Aβ peptides. (Note: With very efficient catalytic antibodies this binding assay may have to be performed on ice to ensure that no cleavage of Aβ occurs during the 1h incubation time.) The assay allows the rapid identification of clones which produce high affinity anti-Aβ antibodies.

Table 6 ^{125}I-$A\beta_{1-40}$ Binding to a Purified Monoclonal Anti-Aβ Antibody *

Addition	^{125}I-$A\beta_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79

* anti-Aβ 5A11 at $2x10^{-6}$ M; Added ~70,000 cpm of ^{125}I-$A\beta_{1-40}$

Monoclonal antibodies from ~~some of~~ hybridoma ~~supernatants~~ obtained using the phenylalanine statine transition state Aβ-KLH antigen were screened ~~using~~ by ELISA to assess their binding to both the normal $A\beta_{1-43}$ peptide and to the phenylalanine statine transition state Aβ peptide. Two major patterns were found (Fig. 13).

One group of antibodies (the left portion of Fig. 13) bound to the immunizing transition state peptide and cross-reacted strongly with the native $A\beta_{1-43}$ peptide (~~when~~ each was adsorbed directly onto the ELISA plate). ~~A~~ The second group (the right portion) showed a high binding preference for the phenylalanine statine transition state Aβ peptide and reacted minimally with native $A\beta_{1-43}$.

Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while Hy media alone or PBS gave a low background (Fig. 13). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, provides a means for ~~choosing~~ identifying monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, ~~the antibodies bound to the~~ experiment was performed with carrier-free Aβ peptides adsorbed directly onto microtitre plates, indicating antibody specificity for the peptide, rather than the carrier.

These findings indicate that several of the generated anti-Aβ transition state antibodies were unique. They bound to both the phenylalanine statine- and normal-Aβ peptides. Their selective recognition of the transition state and weaker cross-reaction with native $A\beta_{1-43}$ however, indicates that this binding interaction is very different from that shown by conventional anti-native Aβ antibodies. It further indicates that these new antibodies may be able to force the native Aβ peptide into a conformation resembling the transition state for hydrolytic cleavage. Importantly, some of the antibodies which showed only

minimal binding to $A\beta_{1-43}$ in this ELISA, did display cross-reactivity with the natural peptide using a highly sensitive ^{125}I-$A\beta_{1-43}$ binding assay (Table 6).

ELISAs were also performed to investigate the binding of anti-statine analog antibodies to both the normal $A\beta_{1-43}$ peptide and to the statine transition state $A\beta$ peptide (Fig. 14). The antibodies bound to the C-terminal locus on these carrier-free $A\beta$ peptides (adsorbed directly to the microtitre plate) confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine $A\beta$ transition state but cross-reacted with native $A\beta_{1-43}$. This indicates that these new antibodies are able to force the native $A\beta$ peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage is predicted to convert $A\beta_{1-43}$ into potentially less harmful shorter peptides, like $A\beta_{1-40}$ or $A\beta_{1-39}$.

Clone 11E9 had the strongest preference for the statine analog and may be the most likely to have catalytic activity (Fig. 14). Several clones displayed no difference in their reactivity with the native versus statine transition state $A\beta$ peptide. The clones were also tested with $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of $A\beta$ (Fig. 14). Used therapeutically, such antibodies should preferentially bind/cleave the less abundant but more noxious $A\beta_{1-43}$ species in the blood, as opposed to the smaller and less detrimental $A\beta_{1-40}$.

Solid phase and TLC $A\beta$ proteolytic assays

A solid phase ^{125}I-labeled $A\beta$ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The peptide Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide (SEQ ID NO: 5) which encompasses amino acids 14-25 of $A\beta$ was radiolabeled and coupled

to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. The product was contacted with anti-transition state antibody and assayed for the progressive release of soluble ^{125}I-peptide from the solid phase matrix. Release of radioactivity from the ^{125}I-Aβ-Sepharose was used to identify catalytic activity (Fig. 15). The assay was verified by the ability of several different proteases to rapidly hydrolyze this Sepharose-linked Aβ substrate. The peptide was readily accessible to proteolytic cleavage as revealed by a release of soluble ^{125}I-peptide that increased with incubation time.

The results presented in Figure 15 indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls. Large amounts of these antibodies can be obtained, purified and tested at higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide this same strategy can be used to assay antibodies reactive with different regions of Aβ.

A thin layer chromatography-based autoradiography assay was devised to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. Two ^{125}I-labeled peptides, ^{125}I-Aβ$_{1-40}$ and a 17-mer, encompassing amino acids 9-25 of Aβ, were used to test for peptide cleavage. The antibodies were added to the ^{125}I-peptides, allowed to incubate and the reaction mix spotted onto polyamide thin layer sheets which were then developed in different solvents. The migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system. Quantitation of the different ~~sized~~ labeled peptide fragments produced indicated that addition of the antibodies to the Aβ

peptides lead to significant break down of the Aβ peptides compared to the untreated peptides (PBS).

Disaggregation of β-amyloid by monoclonal antibodies

The self-aggregation of synthetic Aβ peptides leads to microscopic structures resembling amyloid plaques in the brain (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)) which exhibit the same bright green fluorescence upon exposure to thioflavin T. These aggregates are very stable and usually require harsh detergents or strong acids to dissolve. However, it has been demonstrated that the binding of certain anti-Aβ monoclonal antibodies can effectively inhibit the initial aggregation of this peptide and also disaggregate preformed Aβ complexes (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)).

A radioactive assay was used to quickly screen the different monoclonal antibodies for an ability to dissolve preformed Aβ aggregates made with ^{125}I-labeled and unlabeled soluble Aβ peptide. An aliquot of the labeled aggregate was incubated with either PBS, the 5A11 anti-Aβ antibody, or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor), and the level of released radioactivity was measured (Table 7). The Aβ-specific 5A11 antibody solubilized 80% of the Aβ aggregates while an equal amount of the control antibody had only a minor effect, suggesting that the equilibrium was displaced by antibody-mediated binding of soluble Aβ.

Table 7 Solubilization of ^{125}I-Aβ_{1-40} Aggregate by Monoclonal Anti-Aβ Antibody

Addition	^{125}I-Aβ_{1-40} in Ppt. (cpm)	Amount Solubilized (% of PBS Control)

PBS control	3,420	-
+ 5A11 anti-Aβ	676	80
+ 7D3 anti-TfR	2,458	27

Production of Vectorized Anti-Aβ/Anti-Receptor Bispecific Antibodies

Anti-Aβ antibodies were linked to anti-transferrin receptor antibodies (anti-TfR) which served as vectors for delivery of the anti-Aβ antibodies into the brain. The 7D3 mouse monoclonal antibody was used as the anti-TfR part of the construct. 7D3 is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (Recht et al., *J Neurosurg* 72: 941-945 (1990)). Antibody attachment to the receptor is not blocked by an excess of human transferrin. The epitope recognized by this antibody is therefore distant from the receptor-ligand binding site. Bispecific antibodies constructed with this 7D3 antibody and an anti-Aβ antibody are predicted to be useful for therapy in patients with Alzheimer's disease. Stained with the 7D3 Anti-TfR (Recht et al., *J Neurosurg* 72:941-945 (1990)) and possibly for preclinical trials in primates.

For studies in the transgenic mouse model of Alzheimer's disease an anti-mouse transferrin receptor monoclonal antibody produced in the rat was obtained. This antibody also appears to recognize a transferrin receptor epitope which does not involve ligand binding. The antibody therefore has no effect on cell proliferation when ~~tested~~ using murine lines.

A series of functional assays were performed after completion of the synthesis, purification and size analysis of the anti-Aβ/anti-transferrin receptor bispecific antibodies. The vectorized bispecific antibody, composed of a rat monoclonal antibody directed against the mouse transferrin receptor plus the 5A11 mouse anti-Aβ monoclonal antibody, was tested for the ability to attach to transferrin receptor bearing human cells.

Both components of the bispecific antibody were detected on the cell membrane by cytofluorimetry (Fig. 16) when this duplex was reacted with transferrin receptor positive mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

The capacity of the hybrid reagent to bind ^{125}I-Aβ compared favorably with that of the parent anti-Aβ antibody (Table 8).

Table 8 **^{125}I-Aβ Binding to Bispecific Antibody**

Addition	^{125}I-Aβ_{1-40} Bound (cpm)
Control	4,199
+ anti-Aβ	23,301
+ anti-Aβ/anti-receptor	22,850

To ensure that both of these binding activities resided on the bispecific antibody, transferrin receptor positive cells were treated with the hybrid reagent, unbound material was washed away, and then the cells with bound antibody was exposed to ^{125}I-Aβ_{1-40}. After washing away unbound Aβ, the cell-bound radioactivity was compared to control cells which had been identically prepared except for omission of pretreatment with bispecific antibody. The results are presented in Table 9, and verify the dual specificity of this bispecific antibody by clearly showing that it can simultaneously attach to the cell membrane and bind ^{125}I-Aβ_{1-40}.

Table 9 Bispecific Antibody-Mediated Binding of ^{125}I-Aβ to Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aβ_{1-40} Bound (cpm)
None	2,367
+ anti-Aβ/anti-transferrin receptor	11,476

Transcytosis of bispecific antibody into the brain

A rat monoclonal anti-mouse transferrin receptor antibody was coupled to a mouse monoclonal antibody (obtained from American Type Culture Collection (ATCC TIB 219), also designated R17 217.1.3 (Cell. Immunol. 83: 14-25 (1984))) so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278:1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)).

The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection (Fig. 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma was consistent with its passage across the cerebral endothelial blood-brain barrier (Joachim et al., *Nature* 341: 6239:226-30 (1989)). Even greater accumulation in the parenchyma is expected to occur if the antibodies attach to Aβ in the cerebral plaques of plaque-bearing mice.

Monitoring the brain distribution of bispecific antibody in live mice

The ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live mice would greatly assist in the development of the intracerebral treatment of plaque-bearing mice. Such a development would enable time-course studies and would greatly reduce problems with inter-mouse variability. Preliminary studies with ^{125}I-labeled bispecific antibodies were performed to determine if immunoscintigraphy was feasible in this system. As a first step, either the radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered ~~iv~~ to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. Although this technique suffered from a difficulty in determining how much of the signal was due to the levels of blood-borne radioactivity circulating through the brain, significant distinctions were noted in the brain of mice treated with the mouse transferrin receptor reactive bispecific antibody versus those receiving the control bispecific antibody. When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a much lower level at 24 and 48 hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. The reason for decreased brain levels at 24 hrs and beyond is not known but might be due to dehalogenation of the bispecific antibody probes so that free ^{125}I is released ~~which exits the brain.~~ Alternative methods utilizing radioactive labels such as ^{111}In (Sheldon et al., *Nucl. Med. Biol.* 18:519-526 (1991)) or ^{99m}Tc (Texic et al., *Nucl. Med. Biol.* 22:451-457 (1995)) attached to the vectorized bispecific antibody can be utilized in future experiments if the use of iodine presents a technical problem. This imaging technology will be useful for determining if smaller vectorized bispecific antibodies (eg. F(ab')$_2$) with different physical properties and an altered

biodistribution will penetrate into the brain more effectively.

F(ab')$_2$ heterodimers for vector-mediated transport into the brain

The introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells. The development of smaller vectorized F(ab')$_2$ bispecific reagents is expected to avoid this problem. It has been shown that aggregated Aβ itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect would be greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need to be added.

Faster or more efficient entry into the brain represents another potential advantage that smaller F(ab')$_2$ or Fv$_2$ reagents provide for intracerebral delivery. Such modified bispecific agents can be prepared and compared to full-sized hybrid antibodies for their relative effectiveness in reaching the brain, crossing the blood-brain barrier, and affecting Aβ plaque development, by the methods described herein. It is important to note however that only minor differences were found when the capacity of differently-sized anti-transferrin receptor bispecific reagents for delivering toxins into cells by receptor-mediated endocytosis was compared (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997)). This observation might indicate that little variation will be seen for transcytosis across the brain capillary endothelial cells which form the blood-brain barrier. At the very least however one would expect the two types of vectorized molecules to have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

Materials and Methods

Antigen synthesis. The statine and phenylalanine statine transition state peptides were synthesized using automated Fmoc chemistry. Fmoc-statine (Sta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] and Fmoc-"phenylalanine statine" (PhSta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] were purchased commercially. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. The design strategy and methods for synthesizing phosphonamidate- and phosphonate-based transition state peptides are straightforward (Bartlett et al., *Am. Chem. Society* 22:4618-4624 (1983); Bartlett et al., *Biochemistry* 26:8553-8561 (1987)). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (Bartlett et al., *Am. Chem. Society* 22:4618-4624 (1983); Bartlett et al., *Biochemistry* 26:8553-8561 (1987)) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The last amino acid of Aβ, Thr, was omitted due to potential problems with its unprotected hydroxyl group. The product, Cbz-Gly-PO_2^--NH-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. A reduced peptide bond linkage was placed at the indicated sites in the Aβ molecule. Automated Fmoc chemistry was used to begin synthesis of the peptide. A

pre-synthesized Fmoc amino aldehyde was then added manually and after the imide was reduced, automated synthesis was resumed (Meyer et al., *J. Med. Chem.* 38:3462-3468 (1995)).

Coupling of antigen to carrier. The native and transition state Aβ peptides were coupled to maleimide-activated KLH by standard procedures (Partis et al., J. Pro. Chem 2: 263-277 (1983), in order to elicit an immune response. A Cys residue was strategically placed at the N- or C-terminal end of the peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This stable linkage attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH has been obtained based upon the transition state amino acid content as determined by amino acid analysis of the hydrolyzed conjugates (Tsao et al., *Anal. Biochem. 197*: 137-142 (1991)).

Immunization of mice. Standard protocols were used to immunize the Tg2576 transgenic mice and BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to the hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

Aβ antigens will be emulsified in complete Freunds adjuvant and injected i.p. into BALB/c mice. After ~1 month animals were given a boost i.p. using the antigen emulsified with incomplete adjuvant. Serum from these animals was analyzed for anti-peptide antibodies by ELISA. BALB/c mice showing abundant antibody production were boosted by an i.v. injection with antigen and three days later they were used to generate hybridoma clones that secrete monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of

those induced antibodies cross-react with mouse Aβ and mouse amyloid precursor protein.

Hybridoma production I. A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen. Spleen cells from mice with the highest titre were fused with mouse myeloma NS-1 cells to establish hybridomas according to standard procedures (Köhler et al., *Nature* 256:495 (1975); R. H. Kennett, Fusion Protocols. Monoclonal Antibodies, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol. Plenum Press, New York. 365-367 pp. (1980)).

^{125}I-Aβ binding assay. $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide then separated from unlabeled material by HPLC to give quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89:5462-5466 (1992)). This probe was incubated for 1h at 23°C with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. A polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody. By using serial dilution, this assay can provide relative binding affinities for the different hybridoma supernatants or purified antibodies.

Solid phase Aβ proteolytic assay. A solid phase ^{125}I-labeled Aβ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide (SEQ ID NO:5) encompassing amino acids 14-25 of Aβ was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC. The highly radioactive Aβ peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Antibodies were added to the labeled Aβ, which was then assayed

for progressive release of soluble ^{125}I-peptide from the solid phase matrix at pH 7, 25°C. This assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aβ substrate. Release of soluble ^{125}I-peptide increased with incubation time.

Although Aβ is cleaved by several naturally occurring proteases, preliminary tests indicated that interference from high levels of background hydrolysis was not a problem when assaying hybridoma supernatants of clones that did produce catalytic antibodies. A further precaution that can be taken against exogenous proteases is carrying out all hybridoma cell fusions and cell culturing in serum-free media.

TLC Aβ proteolytic assay. A thin layer chromatography-based autoradiography assay was used to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. The cleavage assay used ^{125}I-Aβ_{1-40} and an ^{125}I-labeled 17-mer, encompassing amino acids 9-25 of Aβ. Binding of the two ^{125}I-labeled peptides to the purified monoclonal antibodies 5A11 and 6E2 was examined using either a PEG precipitation assay or by a co-electrophoresis method. Peptide cleavage was tested by adding the antibodies to the ^{125}I-peptides, incubating and then spotting the reaction mix onto polyamide thin layer sheets. The chromatographs were developed in different solvents (eg. 0.5 N HCl, 0.5 N NaOH or pH 7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system.

Screen and isolate select anti-Aβ antibodies. An ELISA was used to initially screen for anti-Aβ and anti-transition state Aβ peptide monoclonal antibodies. Both the transition state peptide

and the corresponding natural Aβ peptide were adsorbed onto separate microtitre plates. The hybridoma supernatants were screened using two assays so that the relative binding to both native and transition state Aβ peptides could be quantitated. Clones producing monoclonal antibodies that preferentially recognized the transition state or bound Aβ with high affinity were selected for expansion and further study.

Propagation and purification of monoclonal antibodies. Selected clones producing anti-Aβ antibodies and clones producing anti-receptor antibodies were injected into separate pristane-primed mice. Ascites were collected and the specific monoclonal antibodies isolated. Purification of antibodies from ascites was accomplished using a Protein A column or alternatively, antibodies were isolated from ascites fluid by $(NH_4)_2SO_4$ precipitation and passage over an S-300 column to obtain the 150 kDa immunoglobulin fraction. Monovalent Fab fragments were prepared and isolated by established methods. Their purity was evaluated by SDS-PAGE under reducing and non-reducing conditions. 50-100 mg of purified monoclonal antibody was routinely obtained from each ascites-bearing mouse.

Further characterization of catalytic activity on Aβ substrates. To fully define the hydrolytic properties of the isolated anti-transition state antibodies some very important controls can be run. First the ability to completely block catalytic antibody activity with the appropriate transition state peptide can be verified. This non-cleavable "inhibitor" should bind much more tightly to the antibody combining sites and thereby prevent substrate binding or cleavage. Substrate specificity can be further established by showing no cleavage of a sham Aβ peptide having a different amino acid sequence. The products of hydrolysis can also be fully characterized by HPLC, amino acid and mass spectral analysis. Control antibodies that are not

directed against the transition state Aβ can be tested and confirmed to produce no catalysis. Finally, catalytic activity can be shown to reside in the purified Fab fragments of the anti-transition state antibody.

Purified anti-Aβ antibodies dissolve preformed Aβ aggregates. (Walker et al., *Soc. Neurosci. Abstr.* 21:257 (1995), Zlokovic, B.V. *Life Sciences* 59: 1483-1497 (1996)). Aβ precipitates were formed and measured *in vitro* (Yankner et al., *Science* 250: 279-282 (1990), Kowall et al., *Proc. Natl. Acad. Sci.* 88: 7247-7251 (1991)). A radioactive assay was used to quickly screen the different monoclonal antibodies produced for an ability to dissolve preformed Aβ aggregates. After adding ^{125}I-Aβ to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of the labeled aggregate was incubated for 1 hr with either PBS, the 5A11 anti-Aβ antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor). After centrifugation, the level of radioactivity in the precipitate was measured.

Generation of vectorized anti-Aβ/anti-receptor bispecific antibodies. The anti-Aβ antibodies were chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)). A rapid thioether linkage technique was used to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMBP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage

upon mixture with the maleimido-substituted (M) second component following the reaction:

$$Ab_A\text{-}SH + Ab_B\text{-}M \rightarrow Ab_A\text{-}S\text{-}Ab_B$$

Gel filtration of the reaction mixture on an S-300 column yielded the purified dimer which was 300 kDa and had two sites for binding Aβ plus two sites for attachment to transferrin receptors on brain capillary endothelial cells. Non-targeted control hybrids were formed by linking a nonspecific MOPC antibody to the anti-Aβ antibody. This hybrid antibody does bind Aβ, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

$F(ab')_2$ fragments of the two different antibody types can similarly be thioether-linked to form Fc-devoid reagents that cannot bind complement which might otherwise cause neurotoxic effects. These smaller bispecific hybrids (100 kDa) can be formed by reducing the intrinsic disulfides which link the heavy chains of $F(ab')_2$ fragments (Raso et al., *J. Immunol.* 125:2610-2616 (1980)). The thiols generated are stabilized and Ellman's reagent (E) is used to activate these groups on one of the components (Brennan et al., *Science* 229: 81-83 (1985)). Exclusively bispecific $F(ab')_2$ hybrids can be formed upon mixing the reduced Fab' with an activated Fab' having the alternate specificity according to the reaction:

$$Fab'_A\text{-}SH + Fab'_B\text{-}SS\text{-}E \rightarrow Fab'_A\text{-}SS\text{-}Fab'_B + E\text{-}SH$$

Purification on an S-200 column will isolate hybrids with one site for binding Aβ and one site for interaction with the target epitope on the brain capillary endothelial cells.

A similar approach can be used to make even smaller disulfide-linked single chain Fv heterobispecific dimers, Fv_A-SS-Fv_B (50 kDa), to cross the blood-brain barrier. Soluble Fvs can

be constructed to possess a carboxyl-terminal cysteine to facilitate the disulfide exchange shown in the reaction below, and create 50 kDa heterodimers exclusively:

$$Fv_A\text{-}SH + Fv_B\text{-}SS\text{-}E \rightarrow Fv_A\text{-}SS\text{-}Fv_B + E\text{-}SH$$

In side by side comparisons between whole antibody and either Fab' or Fv based bispecific reagents, the latter have proven to be moderately more effective on a molar basis for cell uptake via the transferrin receptor-mediated pathway (Raso et al., J. Biol. Chem. 272: 27623-27628 (1997)). Since these smaller constructs are monovalent for the cell-surface epitope, those findings dispel the notion that cross-linking of two surface receptors is necessary for the cellular uptake of immunocomplexes.

Functional assays for dual binding activity of bispecific antibodies. The capacity of the hybrid reagent to bind ^{125}I-Aβ was compared with that of the parent anti-Aβ antibody in a standard PEG binding assay (see Table 8 for binding assays).

The ability of the appropriate bispecific antibodies to attach to transferrin receptor bearing human or mouse cells was confirmed by cytofluorimetry. The bispecific antibody was reacted with transferrin receptor positive human or mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

Measurement of Aβ binding using ^{125}I-Aβ and a polyethylene glycol separation. To ensure bispecificity, hybrid reagents were tested for a capacity to mediate the attachment of ^{125}I-Aβ to receptor-bearing cells. Transferrin receptor positive cells were treated with the hybrid reagent, washed away unbound material and then exposed these cells to ^{125}I-A$\beta_{1\text{-}40}$. The cells were washed and the amount of cell-bound radioactivity was compared to control cells

which had been identically prepared except that pretreatment with bispecific antibody was omitted.

Capillary depletion. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278:1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)). The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection. Progressive passage from capillaries into the parenchyma indicates active transcytosis across the blood-brain barrier.

Immunoscintigraphy. A non-invasive method for monitoring intracerebral delivery process which involves visualizing the entry of a radiolabeled bispecific antibody into the brain of live mice, can also be used. Radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. The animals were chemically immobilized during exposure using ketamine/xylazine anesthesia. This imaging technology could be very useful for determining if circulating anti-Aβ antibodies will prevent i.v. administered ^{125}I-Aβ from entering the brain. Digital scintigraphy data was quantified using standards and the integration functions provided in the analysis software.

CLAIMS

1. An antibody which catalyzes hydrolysis of β-amyloid at a predetermined amide linkage.

2. The antibody of Claim 1 which catalyzes hydrolysis of the amide linkage between residues X and Y of β-amyloid.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

3. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and also binds to natural β-amyloid with sufficient affinity to detect using an ELISA.

4. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and does not bind natural β-amyloid with sufficient affinity to detect using an ELISA.

5. A vectorized antibody which is characterized by the ability to cross the blood brain barrier and the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage.

6. The vectorized antibody of Claim 5 which is a bispecific antibody.

7. The vectorized antibody of Claim 6 which has a first specificity for the transferrin receptor and a second specificity for a transition state adopted by β-amyloid during hydrolysis.

8. The vectorized antibody of Claim 7 which catalyzes hydrolysis of β-amyloid between residues X and Y.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

9. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
   a) providing antibodies specific for β-amyloid; and
   b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation.

10. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
   a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
   b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

11. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
   a) providing antibodies specific for β-amyloid endogenous to the animal; and
   b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation of the animal.

12. The method of Claim 11 wherein the antibodies specific for β-amyloid are catalytic antibodies which catalyze hydrolysis of β-amyloid at a predetermined amide linkage.

13. The method of Claim 11 wherein the antibodies are monoclonal.

14. The method of Claim 11 wherein the antibodies are polyclonal.

15. The method of Claim 11 wherein the antibodies specifically recognize epitopes on the C-terminus of β-amyloid$_{1-43}$.

16. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

17. The method of Claim 16 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

18. The method of Claim 16 wherein the antigen is ....

19. The method of Claim 17 wherein the antibodies generated have a higher affinity for the transition state analog than for natural β-amyloid.

20. The method of Claim 17 wherein the antibodies generated catalyze hydrolysis of endogenous β-amyloid.

21. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

22. The method of Claim 21 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

23. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is a mimic of a predetermined hydrolysis transition state of a β-amyloid polypeptide endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies to the β-amyloid hydrolysis transition state.

24. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing antibodies which catalyze the hydrolysis of β-amyloid endogenous to the animal; and
    b) intravenously administering the antibodies to the animal.

25. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
    a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and
    b) administering the antibodies to the animal in an amount sufficient to cause a significant reduction in β-amyloid levels in the blood of the animal.

26. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid of the animal at a

predetermined amide linkage; and

b) intravenously administering the antibodies to the animal.

27. The method of Claim 26 wherein the vectorized bispecific antibodies specifically bind the transferrin receptor.

28. The method of Claim 26 wherein the vectorized bispecific antibodies catalyze hydrolysis of the amide linkage between residues X and Y of β-amyloid.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

29. A method for disaggregating amyloid plaques present in the brain of an animal comprising the steps:

a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) intravenously administering the antibodies to the animal in an amount sufficient to cause significant reduction in β-amyloid levels in the brain of the animal.

30. A method for disaggregating amyloid plaques present in the brain of an animal, comprising the steps:

a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) administering the antibodies to the animal.

31. The method of Claim 30 wherein the antibodies are bispecific vectorized antibodies competent for transcytosis across the blood-brain barrier.

32. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:
    a) providing an antigen, the antigen being comprised of an epitope which has a statine analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;
    b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

33. The method of Claim 32 wherein the protein is β-amyloid.

34. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:
    a) providing an antigen, the antigen being comprised of an epitope which has a reduced peptide bond analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;
    b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

35. The method of Claim 34 wherein the protein is β-amyloid.